Personal Care Products Council
Committed to Safety, Quality & Innovation

October 12, 2015

Ms. Michelle Robinson
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Re: Hazard Identification Materials on Diaminotoluenes

Dear Chairman Mack and CIC Members:

The Personal Care Products Council (the Council)[1] appreciates the opportunity to provide comments on the above referenced topic, which were prepared working with Murray and Associates[2]. The Office of Environmental Health Hazard Assessment (OEHHA) asked the Carcinogen Identification Committee (CIC) to determine whether or not diaminotoluenes (mixed), or any of the five individual diaminotoluene isomers not currently listed as causing cancer, should be added to the Proposition 65 list. 2,5-Diaminotoluene and its sulfate salt are widely used as hair dyes, and therefore the review of this isomer by the CIC is of considerable interest to the industry. Our interest is exclusively the 2,5-diaminotoluene isomer; use of any other isomers in hair dyes was discontinued over 40 years ago.

Executive Summary

There is no scientific basis for the listing of 2,5-diaminotoluene as a Proposition 65 carcinogen. Your Committee (the CIC) has been asked to review 2,5-diaminotoluene for listing only because of the confusion surrounding the identity and listing of diaminotoluene (mixed), a commercial mixture of 2,4- and 2,6-diaminotoluene with its own CAS number. 2,4-Diaminotoluene is widely accepted to be carcinogenic, and it was placed on the Proposition 65 list of carcinogens in 1988. In contrast, no regulatory authority has identified 2,5-diaminotoluene as a carcinogen because there is no evidence that it is carcinogenic.

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[1] Founded in 1894, the Council is the national trade association representing the personal care products industry. Our membership includes approximately 300 active member companies that manufacture or distribute personal care products and approximately 300 additional associate members who provide goods and services to manufacturers and distributors of personal care products.
[2] F. Jay Murray, Ph.D., DABT, San Jose, CA

The National Cancer Institute (NCI) conducted long-term carcinogenicity studies of 2,5-diaminotoluene (as the sulfate salt) in mice and rats, and NCI concluded that this substance was "negative" in both sexes of both species. For the reasons detailed herein, NCI did not consider any tumors to be increased due to treatment with 2,5-diaminotoluene. Even if the NCI studies had demonstrated that 2,5-diaminotoluene is carcinogenic (which they clearly do not), it would need to be clearly shown ***through scientifically valid testing according to generally accepted principles***. These studies, while typical of studies conducted by NCI and others in the mid-1970s, would not meet current standards of scientifically valid testing according to generally accepted principles."

While 2,5-diaminotoluene is positive in a number of *in vitro* genotoxicity studies, it is not genotoxic *in vivo*. The Hazard identification Materials indicate that the majority of *in vivo* genotoxicity studies of 2,5-diaminotoluene are negative. However, the Hazard Identification Materials indicate two *in vivo* genotoxicity studies were positive. We respectfully disagree. For the reasons detailed herein, there is no convincing evidence of genotoxicity *in vivo* in any study of 2,5-diaminotoluene.

And finally, it is important to recognize that 2,4- and 2,5-diaminotoluene are completely different in terms of their potential to cause cancer, as summarized in Table 1. Unlike 2,4-diaminotoluene, 2,5-diaminotoluene does not meet the standard of clearly shown through scientifically valid testing according to generally accepted principles to cause cancer.

Table 1. Comparison of the Evidence of Carcinogenicity of 2,4- and 2,5-Diaminotoluene

	2,4-Diaminotoluene	2,5-Diaminotoluene
NCI bioassay: Positive in male rats	Yes[a]	No
NCI bioassay: Positive in female rats	Yes[a]	No
NCI bioassay: Positive in male mice	No	No
NCI bioassay: Positive in female mice	Yes[a]	No
Genotoxic *in vivo*	Yes	No[b]
Genotoxic *in vitro*	Yes	Yes
IARC Classification as a Carcinogen	Yes[c]	No[d]
NTP Report on Carcinogens: Reasonably Anticipated to be a Human Carcinogen	Yes	No
US EPA: B2 Probable Human Carcinogen	Yes	No
European Union: Banned as a hair dye	Yes[e]	No[f]

[a] Multiple tumor types
[b] The Hazard Identification Materials describe mostly negative *in vivo* genotoxicity studies. They also allege the existence of two "positive" *in vivo* genotoxicity studies; we respectfully disagree with this characterization, as discussed herein.
[c] IARC Group 2B
[d] IARC Group 3
[e] Banned; Cosmetic Regulation 1223/2009, Annex II, Ref. 364.
[f] Approved hair dye ingredient; Cosmetic Regulation 1223/2009, Annex III, Ref. 9(a).

I. 2,5-Diaminotoluene (2,5-DAT) is under consideration for listing solely based on re-consideration of diaminotoluene (mixed)

2,5-Diaminotoluene, and other isomers of diaminotoluene, are coming before the CIC because the listing of diaminotoluene (mixed) on Proposition 65 has been questioned. Without the issue of diaminotoluene (mixed), 2,5-diaminotoluene would not have come up for listing consideration because there is no basis for its listing.

Diaminotoluene (mixed) is a well-defined article of commerce, being a starting material in the manufacture of polyurethanes. Diaminotoluene (mixed) consists of a mixture of 2,4-diaminotoluene and 2,6-diaminotoluene, and it has the CAS number 25376-45-8, as listed in the Code of Federal Regulations, 40 CFR §372.65. The mixture, with this CAS number, is listed in the Chemical Book as 2,4/2,6-diaminotoluene[3]. Diaminotoluene (mixed) is carcinogenic because of the presence of the carcinogenic 2,4 isomer. When EPA classified diaminotoluene (mixed) as carcinogenic, it was identified by the CAS number for 2,4-diaminotoluene (95-80-7), and the EPA's assessment was based entirely on data for 2,4-diaminotoluene ("Diaminotoluene is a probable human carcinogen..... [t]his evidence is based on the carcinogenic properties of the isomer 2,4-diaminotoluene.").[4] Neither CAS number 25376-45-8 nor 95-80-7 has any connection with 2,5-diaminotoluene, which has the CAS number 95-70-5.

A comparison of the outcomes of authoritative reviews of 2,4-diaminotoluene and 2,5-diaminotoluene in Table 2 illustrates the clear difference between these two isomers:

Table 2. Outcome of Reviews of 2,4- and 2,5-Diaminotoluene

Reviewing Organization	2,4-Diaminotoluene Outcome	2,5-Diaminotoluene Outcome
IARC	Group 2B, Possibly Carcinogenic in Humans	Group 3, Not Classifiable
NTP Report on Carcinogens (RoC)	Reasonably Anticipated to be a Human Carcinogen	Not Listed
U.S. EPA	B2, Probable Human Carcinogen	Not Classified
European Commission	Banned as a hair dye	Approved hair dye (free base and sulfate salt); favorable opinion from Scientific Committee on Consumer Safety[5]

[3] http://www.chemicalbook.com/Search_EN.aspx?keyword=25376-45-8

[4] U.S. Environmental Protection Agency (1988) Evaluation of the Potential Carcinogenicity of Diaminotoluene (Mixed) (95-80-7) PB93-185270; EPA/600/8-91/103

[5] Scientific Committee on Consumer Safety. Revision on 18 September 2012. Opinion on Toluene-2.5-diamine and its sulfate SCCS/1479/12. http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_093.pdf

II. 2,5-Diaminotoluene (2,5-DAT) is not carcinogenic in mice or rats

The National Cancer Institute (NCI, 1978) concluded that 2,5-diaminotoluene sulfate is "negative" for carcinogenicity

While there are no animal carcinogenicity studies of 2,5-diaminotoluene itself, the NCI conducted a long-term carcinogenicity studies of 2,5-diaminotoluene sulfate, a salt of 2,5-diaminotoluene and sulfuric acid, in Fischer 344 rats and B6C3F1 mice.[6] The NCI did not conclude that 2,5-diaminotoluene sulfate is carcinogenic. To the contrary, NCI concluded: "Under the conditions of this bioassay, sufficient evidence was not obtained to demonstrate the carcinogenicity of 2,5-toluenediamine sulfate in either Fischer 344 rats or $B6C3F_1$ mice."[7] NCI also concluded that 2,5-toluenediamine sulfate was "negative" for carcinogenicity in both sexes of rats and mice. The results of this bioassay by NCI are summarized in the NCI Technical Report No. 126 (TR-126) as follows in Table 3:

Table 3: NCI Bioassay Summary: Levels of Evidence of Carcinogenicity[8]

Sex Species	Results
Male Rat:	Negative
Female Rats:	Negative
Male Mice:	Negative
Female Mice:	Negative

The testicular interstitial cell tumors in male rats are not treatment-related

A statistically significant increase in the incidences of testicular interstitial cell tumors was reported at both the low and high dose levels compared to their respective control groups. However, the NCI did not consider these tumors to be treatment-related:

> "Although the incidence of interstitial-cell neoplasms of the testis was statistically significant in each dosed male rat group, development of these tumors was not considered attributable to compound administration since spontaneous incidence of these neoplasms in male Fischer 344 rats is both high and variable. It should also be noted that control rats were housed in a separate room from dosed rats. There were no other neoplasms occurring in male rats at statistically

[6] National Cancer Institute (1978) Bioassay of 2,5-Toluenediamine Sulfate for Possible Carcinogenicity (CAS No. 6369-59-1) NCI-GG-TR-126.
http://ntp.niehs.nih.gov/results/pubs/longterm/reports/longterm/tr100199/abstracts/tr126/index.html
[7] Id.
[8] Id.

significant incidences, and none of the incidences of neoplasms observed in female rats were statistically significant."[9]

In comparison, the Hazard Identification Materials note:

> "The study authors discounted these tumors based on their high spontaneous incidence in rats, as reported in the literature (Cockrell and Garner, 1976). While it is true that male F344 rats have a high spontaneous incidence of testicular interstitial cell tumors, e.g., 80.5 percent (1445/1794) as reported by Goodman et al. (1979) in NCI Carcinogenesis Testing Program studies conducted from 1972 to 1978, the statistically significant increases observed in this study in both dose groups, as compared to their respective controls, ***suggest*** that these tumors are treatment-related."[10] [emphasis added]

But, the comparison against historical controls mentioned in the above paragraph from the Hazard Identification Materials is not appropriate. First, the historical control data should come from the same laboratory where the 2,5-diaminotoluene sulfate study was conducted, i.e., Mason Research Institute. Goodman et al (1979) report the average incidence of tumors among all studies at all laboratories used by NCI, not just those at Mason Research Institute. Second, according to NCI, "the spontaneous incidence of these neoplasms in male Fischer 344 rats is both high and ***variable***." [emphasis added] Therefore, it is important to consider the range (not just the average) of historical control values observed among the relevant studies. Third, NTP provided historical control data (updated in 1999) for testicular tumors (currently termed testicular adenoma) among 20 NCI/NTP dietary carcinogenicity studies in male Fischer 344 rats given the NIH-07 diet (the same diet used for the study of 2,5-diaminotoluene).[11] The incidence among these historical controls ranged from 74 to 96%, with an average value of 87%; however, the identity of the laboratories and the dates of the studies were not provided, so it is not possible to determine the historical control range for studies conducted at Mason Research Institute.[12]

There is another important consideration in evaluating the incidences of testicular interstitial cell tumors in the bioassay of 2,5-diaminotoluene sulfate. As noted in the Hazard Identification Materials, the survival in the two groups exposed to 2,5-diaminotoluene sulfate exceeded the survival of their respective concurrent control groups as shown in Table 4.

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[9] Id., p. 44.
[10] OEHHA (2015) Hazard Identification Materials. p., 19.
[11] http://ntp.niehs.nih.gov/ntp/research/database_searches/historical_controls/path/r_orlfd.txt
[12] Id.

Table 4. Comparison of survival and incidence of testicular interstitial cell tumors among male rats given 2,5-diaminotoluene sulfate (2,5-DATS) in the diet

Concentration of 2,5-DATS in the diet	0 ppm	600 ppm	0 ppm	2000 ppm
Survival to at least 85 days	58% (29/50)	84% (42/50)	72% (18/25)	90% (45/50)
Testicular interstitial cell tumors	73% (33/45)	90% (43/48)	79% (19/24)	98% (47/48)

As noted by NCI, testicular interstitial cell tumors are common spontaneous tumors that occur at a high and variable incidence among male Fischer 344 rats. The longer a rat survives, the greater the chance of developing a common spontaneous tumor. The differences in survival observed in Table 1 could easily explain the difference in background rates of testicular tumors observed in this study. In every control and dose group, the incidence of testicular tumors is slightly greater than the survival rate. This observation lends further support to NCI's conclusion that "there was no convincing evidence of the carcinogenicity of 2,5-toluenediamine sulfate in rats."[13]

The incidences of alveolar/bronchiolar tumors observed in female mice are not treatment-related

Compared to its concurrent control group, a statistically significant increase in the incidence of alveolar/bronchiolar adenomas and combined alveolar/bronchiolar adenomas and carcinomas was observed among female mice (but not male mice) administered the high dose level of 2,5-diaminotoluene sulfate. However, there was a nearly 4-fold difference in the incidence of combined alveolar/bronchiolar adenomas and carcinomas between the two negative control groups (8.9% vs. 2.3%), suggesting a high degree of variability in the spontaneous occurrence of these lung tumors in mice. In fact, if the two negative control groups had been reversed, there would have been no statistically significant increase in lung tumors among the high dose female mice.

The NCI did not consider the lung tumors among high dose female mice to be treatment-related:

> "The only site of significantly increased tumor incidence among dosed female mice was the lungs. The combined incidence of alveolar/bronchiolar adenomas and alveolar/bronchiolar carcinomas was statistically significant for the high dose group. The combined incidence of these tumors in both high and low dose female mouse groups were elevated relative to historical controls. However, it should be noted that high dose control mice were housed in a separate

[13] NCI (1978), NCI-GG-TR-126, p. 25.

room from dosed mice and received in separate shipments from dosed mice. Because of these factors, this increased incidence does not provide sufficient evidence of a compound-related effect."[14]

Similarly, NCI noted in the Executive Summary:

> "A statistically significant increase in lung tumors in high dose female mice was not considered convincing evidence of a compound-related carcinogenic effect because high dose mice were received in separate shipments from their controls and housed in separate rooms from their controls."[15]

It is also important to note that there was no evidence of any increase in lung tumors in male mice at either dose level.

There is no clear evidence of carcinogenicity of 2,5-diaminotoluene "through scientifically valid testing according to generally accepted principles."

In order to list 2,5-diaminotoluene, it must be "clearly shown ***through scientifically valid testing according to generally accepted principles*** to cause cancer." [emphasis added] The evidence in the NCI carcinogenicity studies fall far short from clearly demonstrating that 2,5-diaminotoluene causes cancer. But, even if the studies had clearly shown that 2,5-diaminotoluene causes cancer (and they do not), clear evidence of carcinogenicity would have to be shown ***through scientifically valid testing according to generally accepted principles.*** Certainly, this is not the case with the NCI carcinogenicity studies.

The actual dates when the NCI study of 2,5-diaminotoluene sulfate was conducted at Mason Research Institute is not provided in the study report. However, the study report itself is dated 1978, which means the study was started no later than the mid-1970s. The study report is identified as NCI-GG-TR-126 in an era when the NCI was in charge of the federal carcinogenicity testing program. At some point after 1978, the responsibility for this program was transferred to the National Toxicology Program (NTP).

The design of the NCI carcinogenicity studies of 2,5-diaminotoluene would not come close to meeting the current requirements of scientifically valid testing according to generally accepted principles. Many of the limitations of these studies are described in the Hazard Identification Materials. For example, there

[14] NCI (1978), NCI-GG-TR-126, p. 44.
[15] Id., p. vii.

were only two dose groups in the study. Each dose group was assigned a "concurrent" control group; however, neither of the concurrent control groups was placed on the study concurrently with their respective dose groups. In fact, the control and dose groups came from different shipments from different suppliers, and they were kept in different rooms. Common generally accepted principles include: all test groups should be run concurrently, all animals should come from the same shipment and from the same supplier, and all animals should be housed in the same animal room. It is a generally accepted principle that all animals on a study should be treated the same in all respects with the exception of differences in the dose levels of test material administered. This was certainly not the case with the NCI carcinogenicity studies of 2,5-diaminotoluene.

The Hazard Identification Materials do not identify other important limitations of the NCI studies of 2,5-diaminotoluene in rats and mice that are inconsistent with scientifically valid testing according to generally accepted principles. For example, the controls and treated groups were not only housed in separate rooms, they were housed in the same rooms as animals from other studies of potent carcinogenic substances. For example, in the Methods section of NCI-GG-TR-126, NCI states that the rats given 2,5-diaminotoluene sulfate in the diet were housed with rats receiving various other test materials (including potent known carcinogens) in the diet:

> "Dosed rats were housed in a room with other rats receiving diets containing acetylaminofluorene (53-96-3); a mixture of dulcin (150-69-6) and L-arginine glutamate (4320-30-3); sodium nitrite (7632-00-0); L-arginine glutamate (4320-30-3); N-butylurea (592-31-4); 2-chloro-p-phenylenediamine sulfate (61702-44-1); N,N-dimethyl-p-nitrosoaniline (138-89-6); 2,4-dinitrotoluene (121-14-2); 4-nitroanthranilic acid (619-17-0); 1,5-naphthalenediamine (2243-62-1); N-(1-naphthyl(ethylenediamine dihydrochloride (1465-25-4); and aniline hydrochloride (142-04-1). Control rats were housed in a room with other rats receiving diets containing 1-nitronaphthalene (86-57-7); 5-nitro-o-toluidine (99-55-8); hydrazobenzene (530-50-7); 2-aminoanthraquinone (117-79-3); 6-nitrobenzimidazole (94-52-0); 3-amino-9-ethylcarbazole hydrochloride; 2,4-diaminoanisole sulfate (615-05-4); and APC (8003-03-0)."[16]

Similarly, mice from the study of 2,5-diaminotoluene sulfate were housed in different rooms with mice receiving other test materials in the diet:

[16] Id., p. 9.

"High dose mice shared a room with other mice receiving diets containing 5-nitro-o-toluidine (99-55-8); hydrazobenzene (530-50-7); 3-amino-9-ethylcarbazole hydrochloride; 1-nitronaphthalene (86-57-7); 6-nitrobenzimidazole (94-52-0); 5-nitro-o-anisidine (99-59-2); and 2,4-diaminoanisole sulfate (615-05-4). High dose control mice were housed in a room with other mice receiving diets containing 2-methyl-1-nitroanthraquinone (129-15-7); 4-chloro-m-phenylenediamine (5131-60-2); acetylaminofluorene (53-96-3); p-cresidine (120-71-8); and fenaminosulf (140-56-7). Low dose mice and their controls were in a room with other mice receiving diets containing amitrole (61-82-5); APC (8003-03-0); N,N-dimethyl-p-nitrosoaniline (138-89-6); 2,4-dinitrotoluene (121-14-2); 4-nitroanthranilic acid (619-17-0); 2-aminoanthraquinone (117-79-3); 3-amino-4-ethoxyacetanilide (17026-81-2); 3-amino-9-ethylcarbazole hydrochloride; l-amino-2-methylanthraquinone (82-28-0); 5-nitro-o-anisidine (99-59-2); 1-nitronaphthalene (86-57-7); 5-nitroacenaphthene (602-87-9); 3-nitro-p-acetophenetide (1777-84-0); and 2,4-diaminoanisole sulfate (615-05-4)."[17]

By today's standards, the NCI carcinogenicity studies of 2,5-diaminotoluene in mice and rats cannot be considered "scientifically valid testing through generally accepted principles" for purposes of identifying 2,5-diaminotoluene sulfate as a carcinogen, even if these studies had provided convincing evidence of carcinogenicity (which they do not).

The results of the NCI studies of 2.5-diamintoluene sulfate do not resemble the clear pattern of carcinogenicity of 2,4-diaminotoluene in NCI studies.

Despite the limitations of the NCI carcinogenicity studies of 2,5-diaminotoluene sulfate, these studies are sufficient to show that 2,5-diaminotoluene does not mimic the carcinogenicity activity of 2,4-diaminotoluene, a substance that is on the Proposition 65 list – and appropriately deserves to be on the Proposition 65 list. Table 5 shows the marked differences between the results of the NCI carcinogenicity studies of 2,4-diaminotoluene and 2,5-diaminotoluene sulfate.

[17] Id., p. 10.

Table 5. Comparison of the NCI Carcinogenicity Studies of 2,4-Diaminotoluene (2,4-DAT) and 2,5-Diaminotoluene sulfate (2,5-DATS)

Species	Sex	Tumor Type	2,4-DAT (1979)	2,5-DATS (1978)
F344 Rat	M	Hepatocellular adenoma and carcinoma	Yes	No
		Mammary gland adenoma	Yes	No
		Subcutaneous fibroma	Yes	No
	F	Hepatocellular adenoma and carcinoma	Yes	No
		Mammary adenoma and carcinoma	Yes	No
		Subcutaneous fibroma	Yes	No
B6C3F1 Mice	M	Alveolar/bronchiolar carcinoma	Yes	No
	F	Hepatocellular carcinoma	Yes	No
		Lymphoma	Yes	No

It is also instructive to compare the levels of carcinogenicity assigned to 2,4-diaminotoluene and 2,5-diaminotoluene sulfate by the NCI (Table 6). NCI determined that the level of evidence of carcinogenicity was "positive" for male rats, female rats and female mice administered 2,4-diaminotoluene in the diet (NCI, 1979). In contrast, NCI determined that the level of evidence of carcinogenicity for 2,5-diaminotoluene sulfate was negative in male rats, female rats, male mice and female mice (NCI, 1978).

Table 6. Comparison of the Levels of Evidence of Carcinogenicity of 2,4-DAT vs. 2,5-DATS in the NCI Carcinogenicity Studies

Sex Species	2,4-DAT (NCI, 1979)	2,5-DATS (NCI, 1978)
Male Rat:	Positive	Negative
Female Rats:	Positive	Negative
Male Mice:	Negative	Negative
Female Mice:	Positive	Negative

In summary, 2,4-diaminotoluene exhibits clear evidence of carcinogenic activity in NCI carcinogenicity studies in mice and rats, as well as in mice and rats in additional carcinogenicity studies performed by others. In contrast, there is no clear evidence of carcinogenicity of 2,5-diaminotoluene sulfate in either mice or rats in NCI carcinogenicity studies. Neither 2,5-diaminotoluene nor 2,5-diaminotoluene sulfate

has been clearly shown through scientifically valid testing according to generally accepted principles to cause cancer.

III. 2,5-Diaminotoluene (2,5-DAT) does not pose a genotoxic hazard.

In vivo genotoxicity test results for 2,5-diaminotoluene are negative

Genotoxicity data covering all of the relevant genetic endpoints have been developed for 2,5-diaminotoluene (free base, sulfate and/or dihydrochloride), both *in vitro* and *in vivo*. There are positive results in the *in vitro* tests, as described in the Hazard Identification Materials. *In vivo* genetic toxicity studies have been conducted on 2,5-diaminotoluene addressing the endpoints of chromosome aberration and aneuploidy; DNA damage; somatic cell mutation; and germ cell clastogenicity. All gave negative results with the exception of a Comet Assay measuring primary DNA damage. This study was performed before current Comet Assay standards were agreed on, and even under these circumstances, the Comet Assay showed consistently negative results in all mouse and rat organs except the rat stomach, likely the result of localized irritation/toxicity.

Under the conditions of the *in vivo* Comet Assay, 2,5-diaminotoluene sulfate did not induce DNA damage in any tissue in mice or in rat colon, liver, kidney, urinary bladder, lung, brain and bone marrow. 2,5-Diaminotoluene sulfate did induce DNA-damage in rat stomach cells after the application of an oral gavage dose. Effects observed only in the stomach are likely due to localized irritation/toxicity. Since no information on histology was provided in this study, localized irritation/toxicity cannot be further assessed.

In 2012, the Scientific Committee for Consumer Safety, the advisory body to the European Commission, reviewed the genotoxicity data base as part of their overall review of 2,5-diaminotoluene and its sulfate salt as hair dyes and came to the following conclusion:[18]

> "Overall, the genotoxicity of toluene-2,5-diamine sulfate is sufficiently investigated for the three types of mutation: gene mutation, structural chromosome mutation and aneuploidy. Toluene-2,5-diamine sulfate is genotoxic *in vitro* inducing gene mutations in bacteria but not in mammalian cells, chromosomal aberrations, and unscheduled DNA-repair synthesis in primary hepatocytes *in vitro*.

[18] SCCS (2012), p. 65-66.

The positive *in vitro* results could not be confirmed in *in vivo* experiments covering the same endpoints. Toluene-2,5-diamine sulfate was negative in two mouse bone marrow micronucleus tests, following oral and i.p. administration and in an *in vivo* UDS test following oral administration. The results of the *in vivo* Comet assay (oral gavage) in mice and rats in all organs evaluated except for the rat stomach may confirm the lack of genotoxic activity of toluene-2,5-diamine sulfate *in vivo*. However, issues with regard to interpretation and validity of the *in vivo* Comet assay in general and of the positive result in the rat stomach in particular remain. In addition, toluene-2,5-diamine sulfate was negative in two dominant lethal assays indicating lack of genotoxic activity in germ cells *in vivo*. The negative results in two *in vivo* mouse spot tests following dermal and ip administration may confirm the lack of potential of toluene-2,5-diamine sulfate to induce gene mutations. As the clastogenic effects found *in vitro* were not confirmed in *in vivo* tests, **toluene-2,5- diamine sulfate can be considered to have no *in vivo* genotoxic potential** and additional tests are unnecessary." [emphasis added]

Table 7 summarizes the *in vivo* genotoxicity results for 2,5-diaminotoluene.

Table 7. In Vivo Genetic Toxicity Data for 2,5-Diaminotoluene

Study type	Genetic endpoint	Result
Mouse bone marrow micronucleus assay	Chromosome aberration, aneuploidy	Negative
In vivo/in vitro UDS Test	DNA damage	Negative
Mouse and rat multiple organ Comet Assay	DNA damage	Negative: Colon, liver, kidney, bladder, lung, brain, and bone marrow (mouse, rat); stomach (mouse) Positive: Stomach (rat)
Mouse Spot Test	Somatic cell mutation	Negative
Dominant Lethal Assay	Germ cell clastogenicity assay	Negative

While the results presented in Table 6 are entirely consistent with the results reported in the Hazard Identification Materials,[19] the Hazard Identification Materials include one additional study which requires further discussion.

[19] OEHHA (2015) Hazard Identification Materials. p. 75.

The murine testicular DNA-synthesis inhibition test does not measure genotoxicity

The Hazard Identification Materials purport to identify a second positive *in vivo* genetic toxicity study (murine testicular DNA synthesis assay).[20] The Hazard Identification Materials describe the results as inhibition of DNA synthesis, "an effect suspected to be related to DNA binding, in the testes in mice".[21] However; reduced DNA synthesis is a marker for cytotoxicity/cytostasis; it is not a marker for genotoxicity. No measurement of DNA binding was made in the study. The murine testicular DNA synthesis assay is a non-standard test measuring a non-standard endpoint; there is no OECD or other guideline for this test, and there is no current use of this assay reported in the literature. A review of the assay published in 1982[22] found that 3/4 mutagens, and 6/6 non-mutagens tested positive for inhibition of DNA synthesis, while also causing hypothermia. Reducing testicular temperature in the absence of chemical treatment also inhibited DNA synthesis. The authors concluded that "the DSI test is not reliable as a screening system for the identification of potential mutagens and carcinogens because of the unspecificity of the parameter measured." Further, the suggestion that the results are relevant to DNA binding is refuted by the other negative 2,5-diaminotoluene *in vivo* genetic toxicity results.

The in vivo genetic toxicity profile for 2,5-diaminotoluene is distinctly different from the profile of 2,4-diaminotoluene

The *in vivo* genotoxicity profile for 2,4-diaminotoluene and 2,5-diaminotoluene are distinctly different, as is made clear in the Hazard Identification Materials[23]. Table 8 summarizes the *in vivo* results for 2,4-diaminotoluene, which are in sharp contrast to the results for 2,5-diaminotoluene, summarized in Table 7. 'Inhibition of DNA synthesis' as measured in the murine testicular DNA synthesis assay is not included in the table for the reasons described above.

[20] Greene et al. (1981) Effect of 4 toluene diamine isomers on murine testicular DNA synthesis. Mutation Research 91: 75-79.
[21] OEHHA (2015) Hazard Identification Materials. p. 74.
[22] Donatsch P, Gurtler J, Matter BE. (1982) Critical appraisal of the 'mouse testicular DNA-synthesis inhibition test' for the detection of mutagens and carcinogens. Mutat. Res. 92(1-2):265-73.
[23] OEHHA (2015) Hazard Identification Materials, Table 16, page 75; Table 13, pages 66-69.

Table 8. In Vivo Genetic Toxicity Data for 2,4-Diaminotoluene

Endpoint	Result
Mutation	Multiple positive studies
DNA Damage	Multiple positive studies
DNA Adduct Formation/ Covalent Binding	Multiple positive studies
Unscheduled DNA Synthesis	One positive/One negative study
Micronucleus	Multiple positive studies
SCEs	Positive

In summary, extensive *in vivo* genotoxicity testing has been conducted on 2,5-diaminotoluene. With the exception of a single organ in a single species in a multi-organ assay, all results are negative. The positive Comet Assay result in rat stomach is likely due to local irritation/toxicity. The lack of *in vivo* genotoxicity provides further evidence that 2,5-diaminotoluene does not meet the standard of clearly shown through scientifically valid testing according to generally accepted principles to cause cancer.

Conclusion

There is no basis for the listing of 2,5-diaminotoluene on Proposition 65. The CIC has been asked to review 2,5-diaminotoluene for listing only because of the confusion surrounding the identity and listing of diaminotoluene (mixed). 2,5-Diaminotoluene does not meet the standard of clearly shown through scientifically valid testing according to generally accepted principles to cause cancer.

Thank you for your attention to these issues.

Sincerely,

Linda Loretz

Linda Loretz, Ph.D., DABT
Director, Safety and Regulatory Toxi